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Mechanism of the Antimicrobial Action of Pyrithione: Effects on Membrane Transport, ATP Levels, and Protein Synthesis

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Pyrithione is a general inhibitor of membrane transport processes in fungi. A brief preincubation of *Penicillium* mycelia with pyrithione resulted in a marked decrease in the activities of a variety of independently regulated transport systems, including those for inorganic sulfate, inorganic phosphate, methylamine (actually, the NH_4^+ permease), choline-*O*-sulfate, glucose, L-methionine (a specific system), and several hydrophobic L-α-amino acids (the general amino acid permease). The degree of inhibition at any fixed pyrithione concentration and exposure time increased as the pH of the incubation medium was decreased. This result strongly suggests that the active species is the un-ionized molecule and that pyrithione acts by collapsing a transmembrane ΔpH driving force. The degree of transport inhibition caused by a given concentration of pyrithione increased with increasing time of exposure to the inhibitor. However, exposure time and pyrithione concentration were not reciprocally related. At "low" pyrithione concentrations, transport inhibition plateaued at some finite value. This observation suggests that the fungi can detoxify low levels of the inhibitor. The concentration of pyrithione required for a given degree of growth inhibition increased as the experimental mycelial density increased. This phenomenon was consistent with the suggestion that the fungi are capable of inactivating pyrithione.

Pyrithione [omadine, 2-mercaptopyridine-*N*-oxide, 1-hydroxy-2(1H)-pyridinethione, 2-pyridinethiol-1(*N*) oxide], a derivative of the naturally occurring antibiotic aspergillic acid (15, 16), is a potent antimicrobial agent (13, 21). The sodium salt is reported to be fungistatic at 1 μg/ml (ca. 10^{-5} M) or less, and fungicidal at 100 μg/ml (ca. 10^{-3} M) or less (12). The zinc salt is an effective antidandruff compound (e.g., Head and Shoulders shampoo). The mode of action of pyrithione is unknown. Cooney (5) suggested that pyrithione might act as a nicotinic acid or pyridoxal antimetabolite. However, there has been no experimental evidence published that supports this suggestion.

Cotton (Ph.D. thesis, University of Texas, Austin, 1963) suggested that pyrithione may act by chelating metal cofactors of enzymes. Earlier, Lott and Shaw (11) proposed the same mode of action for the related oxygen analogs (hydroxamic acids). Pyrithione does, in fact, inhibit alcohol dehydrogenase (a zinc-requiring enzyme) (Cotton, Ph.D. thesis). A third obvious possibility is that pyrithione acts as a nonspecific reductant, cleaving disulfide bonds in a variety of proteins.

It occurred to us that the primary effect of pyrithione might be as an inhibitor of membrane transport. This idea was based on the fact that pyrithione is a weak acid (pK_a = 4.7), and we had previously shown that a variety of lipid-soluble weak acids were transport inhibitors at pH values in the region of their pK_a and below (10). The weak acids presumably act by diffusing across the plasma membrane and ionizing intracellularly, thereby collapsing a transmembrane ΔpH.

The experiments reported in this paper were designed to measure the effects of pyrithione on a variety of well-characterized transport systems in two *Penicillium* species (1-4, 6, 7, 9, 10, 19, 22). In addition, the effects of the inhibitor on internal ATP levels and in vivo protein synthesis were determined.

MATERIALS AND METHODS

Growth of mycelia. The experiments described in this paper were carried out with *Penicillium chrysogenum* ATCC 24791 (a wild-type strain) and *Penicillium notatum* ATCC 32337 (a mutant strain lacking ATP sulfurylase but possessing the sulfate and choline-*O*-sulfate transport systems). The organisms were grown aerobically, in submerged cultures, at room temperature (about 25°C) in citrate no. 3 synthetic medium containing (per liter): glucose or sucrose (40 g), $(NH_4)_2H$ citrate (10 g), $(NH_4)_2HPO_4$ (6 g), K_2HPO_4 (16 g), and trace metals (10 ml) (22). The sulfur source added for *P. chrysogenum* was Na_2SO_4 (1 g/liter); for *P. notatum* it was L-cysteic acid (0.1 g/liter). The sugar was sterilized separately and added to the rest of the medium just before inoculation. The pH of the medium was 7.0 at the start and dropped to about 5 after 2 days of growth. The cells were grown in 500-ml Erlenmeyer flasks, containing 100 ml of medium, on a New Brunswick Gyrotory shaker operating at a speed of about 250 rpm and describing a 1-inch (ca. 25.4-mm) circle. After 1 to 2 days of growth the mycelium achieved a density of 3 to 6 g (wet pressed weight) per 100 ml of medium.

Derepression of transport systems. To derepress and deinhibit the various transport systems, the mycelia were incubated in the appropriate nutrient-deficient medium.

Mycelium grown for 2 days on the low-cysteic acid medium described above was fully derepressed for the sulfur-regulated permeases for inorganic sulfate (4, 6, 19, 22) and choline-*O*-sulfate (1). Alternatively, *P. chrysogenum* could be grown on the high-sulfate medium, filtered, washed, and then sulfur starved for 6 to 12 h at a density of 2 g (wet weight) per 100 ml in citrate no. 3 medium minus a sulfur source.

The general amino acid transport system of *P. chrysogenum* was derepressed by incubating the mycelium in citrate no. 3 medium lacking the sugar. (The organism cannot utilize the citrate for growth.) The general amino acid transport system could also be derepressed and deinhibited by incubating the mycelium at 1 to 2 g per 100 ml in a nitrogen-free medium containing (per liter): Na_3 citrate$\cdot 2H_2O$ (14.7 g), Na_2SO_4 (1 g), glucose or sucrose (40 g), and trace metals solution (10 ml), all in 0.1 M potassium phosphate buffer (pH 7.0). The sugar was sterilized separately. Nitrogen starvation also promotes the appearance of the methylamine (ammonium ion) transport system (7).

Phosphate-deficient medium (used to derepress and deinhibit the phosphate transport system) contained (per liter): $(NH_4)_2H$ citrate (10 g), NH_4Cl (5.3 g), Na_2SO_4 (1 g), and glucose or sucrose (40 g), adjusted to pH 7.0 with KOH.

Permease assays. After the desired starvation period, the mycelium was removed from the medium and washed three times each with 250-ml volumes of deionized water, potassium phosphate buffer (0.02 M, pH 6.0), deionized water, Na_2 ethylenediaminetetraacetic acid (1.0 mM, pH 6.0), and, finally, again with deionized water. The mycelium was filtered, pressed to remove excess moisture, and resuspended at a density of 1.2 g (wet weight) per 80 ml of assay buffer (containing the desired concentration of pyrithione or other potential inhibitor). The standard assay buffers used were 0.05 M or 0.07 M potassium phosphate or 0.02 M 2-(*N*-morpholino)ethane sulfonic acid-tris(hydroxymethyl)aminomethane (adjusted as desired to pH 4.0 to 7.0). The suspension was aerated on a rotary shaker for at least 5 min prior to beginning the assays. Transport rates were calculated from four 2- to 3-ml samples taken at 15-s intervals within the first minute after the addition of labeled substrate. The samples were filtered with suction, and the resulting mycelial pad was placed in a scintillation vial containing 1.0 ml of water and 3 ml of PCS scintillation fluid (Amersham/Searle). All transport rates (micromoles per gram per minute) are reported on a dry-weight basis. One gram (wet weight) of mycelium is equivalent to 0.138 g (dry weight) of cells.

ATP determinations. Samples (4-ml) of the mycelial suspension (containing 60 mg [wet weight] of cells) were removed from samples prepared for permease assays. The suspension was filtered rapidly and washed with 3 ml of ice-cold deionized water, and the resulting pad was placed in 2 ml of 50% EtOH at 80°C for 6 min, then cooled on ice for 15 min. The samples were centrifuged in a clinical table-top centrifuge to remove insoluble cell debris. The supernatant fluid was kept at −20°C until assayed for ATP. The ATP content of the extract was measured fluorometrically at 30°C by means of a hexokinase plus glucose 6-phosphate dehydrogenase-coupled assay (20). The assay measures the increase in the fluorescence caused by reduced nicotinamide adenine dinucleotide phosphate. The amount of reduced nicotinamide adenine dinucleotide phosphate formed is directly proportional to the amount of ATP present. A Turner model 111 fluorometer coupled to a Texas Instrument model PRR recorder was used. The primary filter was Turner 110-811(7-60); the secondary filter was 110-816(2A). Generally, the assay mixture consisted of 2.0 ml of triethanolamine-hydrochloride buffer (pH 7.4), containing 5 mM disodium ethylenediaminetetraacetic acid and 10 mM $MgCl_2$, to which were added 10 μl of 1 M glucose, 10 μl of 0.03 M oxidized nicotinamide adenine dinucleotide phosphate, 10 μl of glucose 6-phosphate dehydrogenase (30 U/ml), and 10 μl of the cell-free ethanolic extract. The mixture was incubated for 2 to 3 min to use up any endogenous glucose 6-phosphate. When the base line was stable, 10 μl of hexokinase (300 U/ml) was added, and the resulting increase in fluorescence was recorded. The system was precalibrated with ATP solutions of known concentrations. A full-scale deflection was equivalent to ca. 5×10^{-7} M ATP in the assay mixture.

Measurements of in vivo protein synthesis. *P. chrysogenum*, nutrient starved for 12 h, was resuspended in 0.05 M potassium phosphate buffer (pH 6.0) at a density of 0.3 g (wet weight) per 20 ml. Labeled substrate was added (3 mM $Na_2{}^{35}SO_4$ or 0.1 mM [^{14}C]tryptophan; specific activity, 1×10^6 to 3×10^6 cpm/μmol), and cultures were incubated on a rotary shaker. After 5 min the mycelium was filtered, washed with deionized water, and resuspended in fresh buffer (0.05 M potassium phosphate, pH 6.0) containing 0.4% (wt/vol) glucose. At various times, two 3-ml samples were removed and filtered by suction. One mycelial pad was washed twice with 5 ml of deionized water and placed in a scintillation vial. The second pad was placed in 5 ml of water at 100°C for 2 min. It was then filtered and washed, and the resulting insoluble pad was placed in a scintillation vial.

Chemicals. Pyrithione (2-mercaptopyridine-*N*-oxide), pyridine-*N*-oxide, 2,4-dihydroxypyridine, 3-hydroxypyridine-*N*-oxide, and dimethyldithiocarbamic acid were purchased from Aldrich Chemical Co. Sodium pyrithione and pyrithione disulfide were generous gifts from Procter and Gamble Co.

Carrier-free $^{35}SO_4^{2-}$ was obtained from Schwarz/Mann. Carrier-free $^{32}P_i$ was obtained from New England Nuclear. These were mixed with unlabeled K_2SO_4 and potassium phosphate buffer, respectively, to obtain solutions of the desired final concentration and specific activity. The stock solutions of [^{14}C]methylamine-hydrochloride (Schwartz/Mann), [*U*-^{14}C]glucose (Amersham/Searle), and the ^{14}C-labeled amino acids (ICN) were prepared similarly.

RESULTS

Growth inhibition. Preliminary experiments established that the two *Penicillium* species used for our studies were susceptible to pyrithione. At pH 7.0, complete inhibition of *P. notatum* growth in submerged culture (starting from spores or a small mycelial inoculum) was obtained with 3×10^{-6} M pyrithione, whereas less than 10^{-7} M pyrithione had no effect. (In 24 h the control mycelial inoculum increased in wet weight from 0.2 g to 4.6 g.) *P. chrysogenum* showed a similar susceptibility.

Effect of pyrithione concentration on transport. Figure 1 shows the dose-response curves at pH 6.0 for the inhibition by pyrithione of $^{35}SO_4^{2-}$ transport in *P. notatum* and L-[^{14}C]-phenylalanine transport in *P. chrysogenum*. The mycelia were preincubated with the pyrithione for exactly 5 min prior to measurement of transport activity. Under the standard assay conditions, 50% inhibition was achieved at about 6×10^{-4} M pyrithione. By way of comparison, Table 1 lists the inhibitor concentrations reducing transport activity to 50% of controls under the same assay conditions for a variety of other antifungal agents that inhibit membrane transport (10).

The large discrepancy between the concentration of pyrithione required for inhibition of growth and the concentration required for inhibition of transport is more apparent than real. A number of experimental conditions are different for the two types of study. For one thing, the dose-response curve shown in Fig. 1 was obtained after a 5-min exposure to pyrithione, whereas, in the preliminary growth inhibition

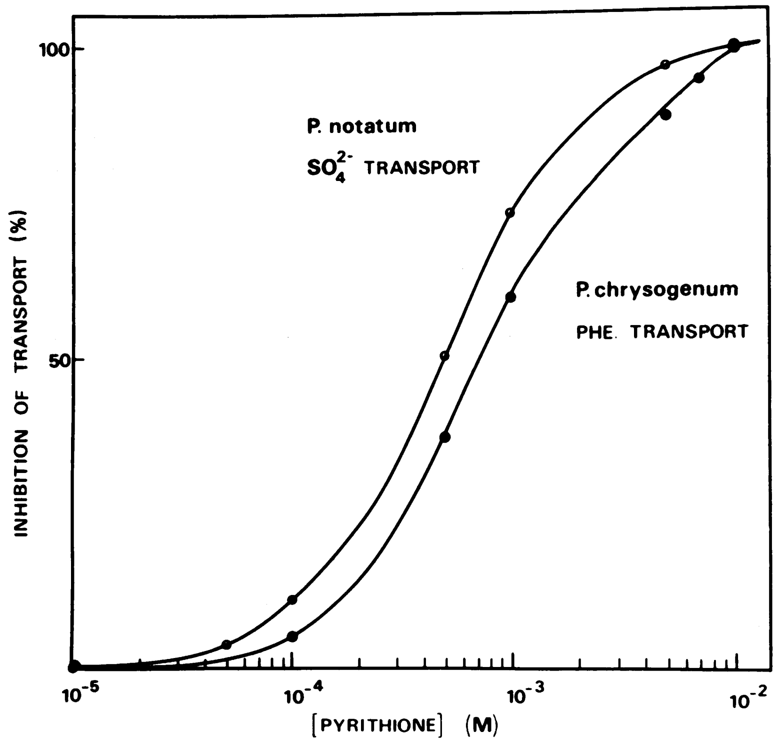


FIG. 1. *Dose-response curve: effect of varying concentrations of pyrithione on the transport of $^{35}SO_4^{2-}$ by low-cysteic acid-grown (i.e., sulfur-deficient) mycelium of P. notatum and on the transport of L-[^{14}C]phenylalanine by nitrogen-deficient mycelium of P. chrysogenum. The transport assays were run at 25°C in 0.07 M potassium phosphate buffer (pH 6.0) containing 0.4% (wt/vol) glucose. The mycelia (0.02 g [wet weight] per ml) were preincubated with the indicated concentrations of pyrithione for 5 min prior to adding the labeled substrate (6.67×10^{-5} M $^{35}SO_4^{2-}$, specific activity, 5×10^7 cpm/μmol or 6×10^{-4} M L-[^{14}C]phenylalanine, specific activity 2×10^5 cpm/μmol). The control $^{35}SO_4^{2-}$ transport rate was 2 μmol $\times g^{-1} \times min^{-1}$; the control L-[^{14}C]phenylalanine transport rate was 5 μmol $\times g^{-1} \times min^{-1}$ (dry-weight basis).*

study, the cells were continually exposed to the pyrithione during the 24-h incubation period. Additionally, the mycelial densities used in the two types of experiment were different: 0.1 to 0.2 g (wet weight) of inoculum per 100 ml for growth inhibition studies versus 2.0 g (wet weight) of mycelium per 100 ml for transport studies. Both factors influence the sensitivity of the organism (see below).

Effect of exposure time on transport. The degree of inhibition caused by a given concentration of pyrithione at a fixed pH and mycelial density depended on the length of time that the mycelium was exposed to the inhibitor. Figure 2 shows the effect of preincubation time with 10^{-3} M pyrithione at pH 6.0 under standard assay conditions (2.0 g [wet weight] of mycelium per 100 ml). A 2- to 4-min exposure was sufficient to yield 50% inhibition of L-[^{14}C]phenylalanine transport; after 20 min, transport activity was reduced by at least 90%. The relatively slow, time-dependent inhibition eliminated from consideration any simple reversible mechanism of inhibition (e.g., competition by pyrithione with the substrate for the permease).

Pyrithione concentration and exposure time were not related in a strict reciprocal manner. For example, under standard transport assay conditions, 3×10^{-5} M pyrithione did not depress $^{35}SO_4^{2-}$ transport by more than 50 to 60%, even after an hour of preincubation with the inhibitor. The effect of 10^{-3} M pyrithione on $^{35}SO_4^{2-}$ transport was essentially identical to that shown in Fig. 2 for L-[^{14}C]phenylalanine transport.

Effect of mycelial density on growth inhibition. Figure 3 shows the effect of initial mycelial density on the concentration of pyrithione required for inhibition of further growth. Clearly, the mycelial density influenced the susceptibility of the culture. At the usual inoculum density (ca. 0.1 to 0.2 g [wet weight] or less per 100 ml), complete growth inhibition could be achieved with less than 10^{-6} M pyrithione. At

TABLE 1. *$[I]_{0.5}$ values for the inhibition of $^{35}SO_4^{2-}$ and L-[^{14}C]phenylalanine transport by a variety of antifungal agents*[a]

Inhibitor[b]	$[I]_{0.5}$ for $^{35}SO_4^{2-}$ transport (M)	$[I]_{0.5}$ for L-[^{14}C] phenylalanine-transport (M)
Pyrithione	4×10^{-4}	8×10^{-4}
Sorbate	7×10^{-4}	5×10^{-3}
Propionate	5×10^{-3}	9×10^{-3}
Benzoate	1×10^{-4}	6×10^{-4}
Salicylate	1×10^{-2}	1×10^{-2}

[a] $[I]_{0.5}$ represents the concentration of inhibitor that reduced transport activity to 50% of the control level. Transport was measured in 0.07 M potassium phosphate buffer (pH 6.0) with 10^{-5} M $^{35}SO_4^{2-}$ (*P. notatum*) or 10^{-4} M L-[^{14}C]phenylalanine (*P. chrysogenum*).

[b] Mycelia were preincubated for 5 min with various concentrations of the inhibitor prior to adding the labeled substrate.

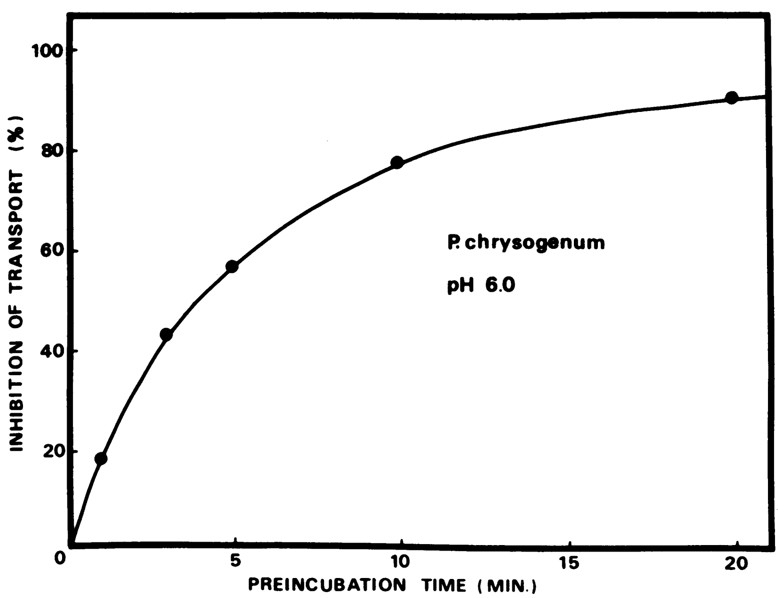


FIG. 2. *Effect of exposure time on L-[^{14}C]phenylalanine transport by P. chrysogenum. The mycelia (grown as described in Fig. 1) were preincubated at a density of 0.02 g (wet weight) per ml with 10^{-3} M pyrithione in 0.07 M potassium phosphate buffer (pH 6.0) for the indicated times. Transport was measured at 10^{-4} M L-[^{14}C]phenylalanine (specific activity, 10^5 cpm/μmol). The control rate was 5 μmol × g^{-1} × min^{-1}.*

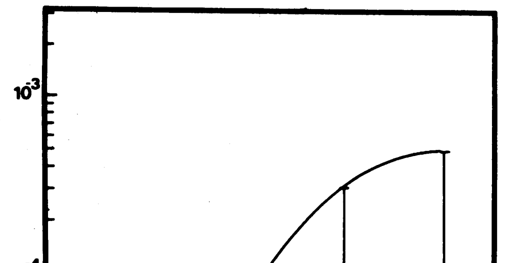


FIG. 3. *Effect of mycelial density on the sensitivity of P. chrysogenum to growth inhibition by pyrithione. The standard growth medium (adjusted to pH 6.0 with concentrated HCl) was inoculated with known weights of mycelium and incubated in the presence of various pyrithione concentrations at 25°C. After 24 h the mycelium was collected by filtration, washed, dried, and weighed. The upper limit of each bar represents the concentration of pyrithione that completely inhibited further growth. The lower limit represents the concentration below which no inhibition was observed.*

the usual transport assay density (2 g/100 ml), nearly 10^{-4} M pyrithione was required; less than about 10^{-5} M pyrithione had no effect. Pyrithione at 3×10^{-5} to 7×10^{-5} M inhibited growth by about 50% when the inoculum size was 2 g/100 ml and the starting pH was 6.0. This same concentration inhibited transport by about 50% at pH 6.0. The results suggest that transport inhibition could be the sole mode of action of pyrithione in inhibiting the growth of fungi. However, secondary long-term effects (e.g., cleavage of protein disulfide bonds) certainly cannot be excluded.

A more thorough comparison of the concentration dependence of growth inhibition and transport inhibition was technically unfeasible. Ideally, the effect of pyrithione on the specific growth rate constant should be determined at different mycelial densities and a constant pH. In the growth study shown in Fig. 3, the initial pH of the medium was 6.0, but this dropped in some cultures to 4.7 to 5.0 during 24 h of incubation. The plot shown is valid only for the specific growth conditions employed. In a medium of different initial pH and different buffer capacity, the concentration of pyrithione required to completely inhibit further growth at any given mycelial density may well be different from that shown. Similarly, since growing cells produce more acid than nongrowing cells, the length of each concentration bar will vary with the buffer capacity of the medium. Since pyrithione can be oxidized to an inactive disulfide, the degree of aeration of the culture and the length of the assay period will also influence the test organism's apparent sensitivity in long-term growth tests.

Effect of pH on transport. If pyrithione acts solely as a nonspecific protein disulfide reductant (thereby denaturing cell surface and/or membrane transport proteins), we would expect the degree of inhibition of transport to increase as the pH of the incubation medium increases. (Increasing the pH of a buffered medium would shift the equilibrium pyrithione-SH$\rightleftharpoons$pyrithione-S^- + H^+ to the right, thereby increasing the concentration of the active pyrithione-S^- species.) However, as shown in Fig. 4, pyrithione becomes more potent as the pH decreases. A 5-min preincubation with 10^{-3} M pyrithione at pH 7.0 reduced L-[^{14}C]phenylalanine transport by only 10 to 20%. At pH 5.0, transport was inhibited 90% after a 5-min exposure. The transport of $^{35}SO_4^{2-}$ by *P. notatum* was affected identically. These results argue against protein disulfide reduction as the primary mode of action of pyrithione on membrane transport. The results clearly show that the active species of pyrithione is the un-ionized molecule.

Effect of pyrithione on cellular ATP levels. Exposure to pyrithione resulted in a marked decrease in the internal concentration of ATP (Fig. 5). The rate at which the ATP level fell and the ultimate level was achieved depended on the concentration of pyrithione and the particular fungal strain. It is noteworthy that the decrease in transport activity was not directly correlated with the decrease in ATP level. For example, with *P. notatum* at pH 5.0, a 2-min exposure of 10^{-3} M pyrithione (in the absence of glucose) reduced $^{35}SO_4^{2-}$ transport activity by more than 90%, while the ATP level was reduced by only about 10%. With *P. chrysogenum* at pH 5.0, 2.5×10^{-3} M pyrithione reduced the ATP

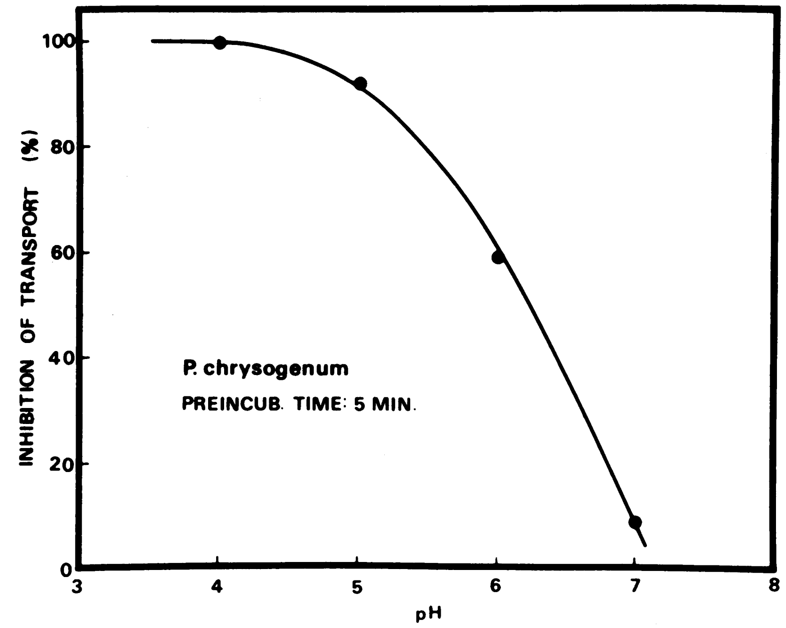


FIG. 4. *Effect of pH on the inhibition of* L-[^{14}C]*phenylalanine transport by P. chrysogenum. The mycelia were preincubated with* 10^{-3} *M pyrithione for 5 min in 0.07 M potassium phosphate buffers of the indicated pH values. The desired pH of the assay medium was obtained by mixing 0.07 M solutions of* KH_2PO_4 *and* K_2HPO_4 *containing 0.4% (wt/vol) glucose. Although phosphate has no significant buffer capacity below pH 6, the pH of the mycelial suspension remained constant over the 5-min preincubation period plus the 1-min assay period. Transport was measured as* 10^{-4} *M* L-[^{14}C]*phenylalanine (specific activity,* 10^5 *cpm/μmol). The percent inhibition is reported relative to control rates, which varied with pH (3).*

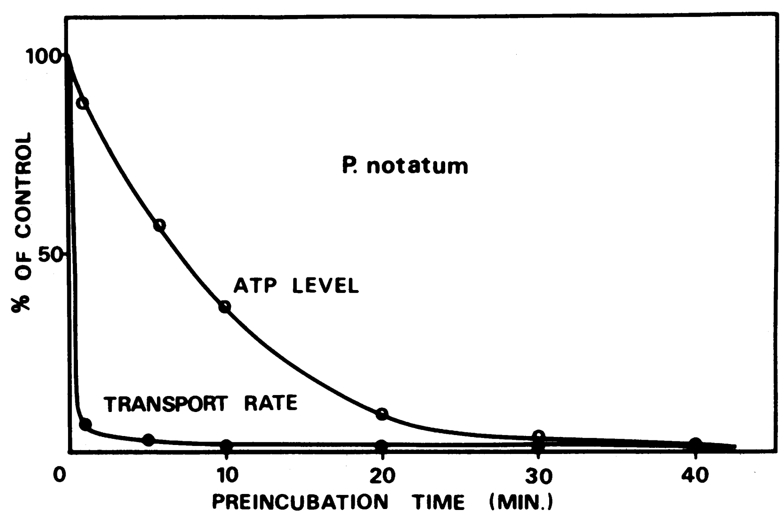


FIG. 5. *Effect of pyrithione on sulfate transport activity and internal ATP levels in low-cysteic acid-grown mycelia. The mycelia were incubated at a density of 0.015 g (wet weight) per ml with* 10^{-3} *M (P. notatum) or* 2.5×10^{-3} *M (P. chrysogenum) pyrithione in 0.05 M potassium phosphate (pH 5.0) without glucose. Samples were withdrawn periodically for the measurement of internal ATP levels (see the text) and* $^{35}SO_4^{2-}$ *transport activity.* $^{35}SO_4^{2-}$ *transport was measured at* 10^{-4} *M (specific activity,* 2×10^6 *cpm/μmol). The rates prior to incubation with pyrithione were 4.2 μmol* $\times$ g^{-1} $\times$ min^{-1} *(P. notatum) and 1.6 μmol* $\times$ g^{-1} $\times$ min^{-1} *(P. chrysogenum). The initial internal ATP levels were* 8×10^{-4} *M (P. notatum) and* 10^{-3} *M (P. chrysogenum). These internal concentrations assume that all the cellular water (86% of the wet weight) is internal and available for solution of the ATP.*

level to about 40% of the initial level within 2 min. But during the same period, L-[^{14}C]phenylalanine transport activity was reduced to about 15% of the initial value. The results agree with our earlier report (10) showing no consistent relationship between inhibition of amino acid transport and reduction in internal ATP levels.

Effect of pyrithione on protein synthesis. Pyrithione prevented the incorporation of internal (accumulated) L-[^{14}C]tryptophan and $^{35}SO_4^{2-}$ into protein (Fig. 6, data for $^{35}SO_4^{2-}$ not shown). The inhibition might simply reflect the diminished supply of ATP. The excretion of the ^{14}C-labeled carbon chain (α-keto acid) by the control mycelium preloaded with L-[^{14}C]tryptophan is normal and is observed for a variety of hydrophobic amino acids transported by the nitrogen-regulated general amino acid transport system of *P. chrysogenum* (9). The inhibition of excretion concomitant with the inhibition of protein synthesis is unexpected.

Structural requirements for inhibition. Experiments with a variety of pyridine derivatives including 2-mercaptopyridine, pyridine-*N*-oxide, 3-hydroxypyridine-*N*-oxide, 2,4-dihydroxypyridine, and pyrithione disulfide clearly established that both the *N*-oxide and the thiol group are required for transport inhibition. The inactivity of 2-mercaptopyridine and also of mercaptoethanol, and dimethyldithiocarbamic acid suggested that the primary inhibitory effect of pyrithione did not result from the reduction of disulfide bonds in cell surface proteins.

Transport systems susceptible to pyrithione. For convenience, the preceding experiments were restricted to the sulfate transport system of *P. notatum* and the general amino

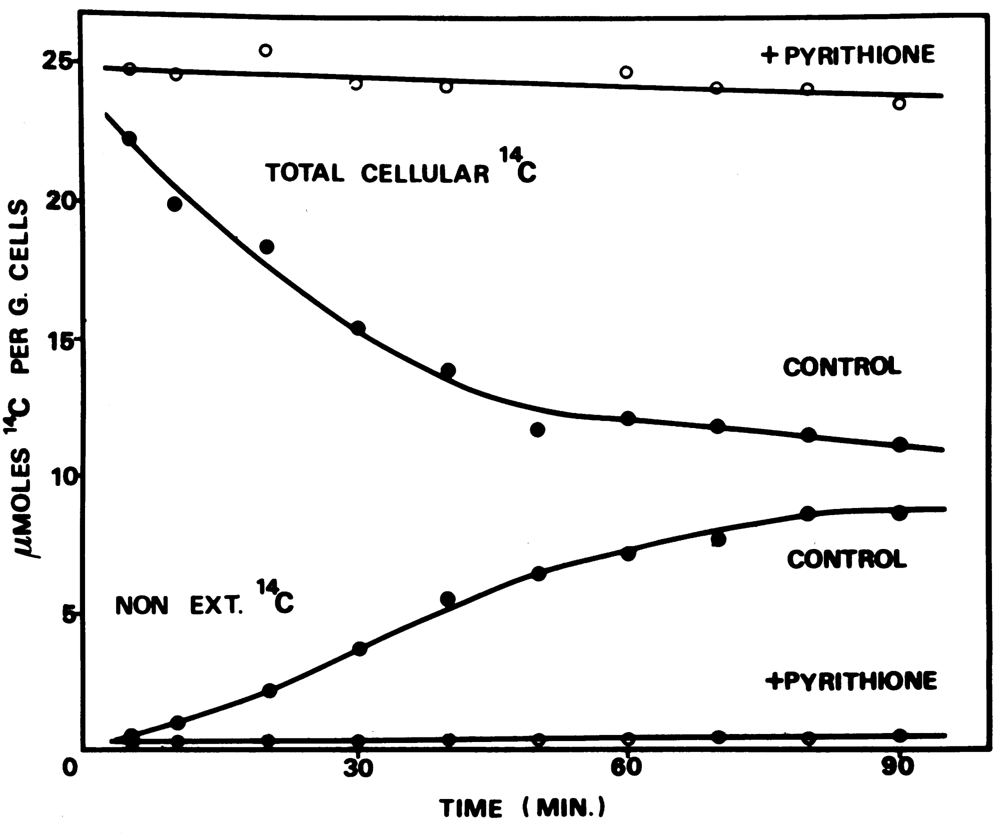
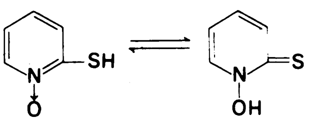


FIG. 6. *Effect of pyrithione on the incorporation of accumulated* L-[^{14}C]*tryptophan into protein. Nitrogen-deficient mycelium was preincubated for 5 min at a density of 0.015 g (wet weight) per ml in 0.05 M potassium phosphate buffer (pH 6.0) containing 10^{-4} M* L-[^{14}C]*tryptophan (specific activity, 1.6×10^6 cpm/µmol). The mycelia were then filtered, washed with buffer, and resuspended at the original density in fresh buffer containing 0.4% (wt/vol) glucose, without and with 5×10^{-3} M pyrithione. The total mycelial radioactivity and the insoluble radioactivity were determined as described in the text. The nonextractable radioactivity was assumed to represent ^{14}C incorporated into protein. Incorporation is reported on a dry-weight basis.*

acid transport system of *P. chrysogenum* (with L-[^{14}C]phenylalanine as the substrate). However, the results in Table 2 demonstrate that pyrithione inhibits a wide variety of membrane transport systems. The lack of specificity suggests that the inhibitor affects some factor common to all active transport systems.

DISCUSSION

In solution, pyrithione exists as a mixture of two tautometric species:



Both the *N*-oxide group and the thiol group are required for inhibition of transport. This combination renders the pyridine derivative significantly more acidic than either parent compound. (The pK_a of pyrithione is about 4.7, compared to >8 for pyridine thiol or pyridine-*N*-oxide and >6 for 3-hydroxypyridine-*N*-oxide.) In spite of the requirement for a moderately strong acid, the active species is the un-ionized molecule (Fig. 4). The results strongly suggest that pyrithione acts as a proton conductor: the uncharged molecule diffuses across the cell membrane and ionizes intracellularly, thereby collapsing a transmembrane ΔpH driving force (8, 10).

Pyrithione also promotes a decrease in the cellular ATP level. This, of course, raises a question concerning the primary action of pyrithione. Is transport inhibited because the cellular ATP level falls? Or are the decreases in transport activity and ATP levels parallel results of a single mode of action? There is little doubt that the ΔpH driving force for transport in fungi is generated by a proton-translocating, membrane adenosine triphosphatase (17, 18), so we can state a priori that anything that reduces cellular ATP production will also reduce transport activity. On the other hand, if both mitochondria ATP production and plasma membrane solute transport are driven by a transmembrane ΔpH, then a proton conductor uncoupler will inhibit both processes. Thus, the question of the primary action of pyrithione may be meaningless. However, in a temporal sense, membrane transport activity falls more rapidly than ATP levels in cells exposed to pyrithione (Fig. 5). The inhibition of in vivo protein synthesis by pyrithione (Fig. 6) very likely results from a diminished supply of ATP.

At first glance, the idea of correlating internal ATP levels with transport activity may seem to be a good approach to deciding whether ATP directly energizes active transport. However, the results (either positive or no correlation) can be inconclusive and even misleading. Consider two cases: (i) ATP is directly involved; the K_m of the membrane adenosine triphosphatase is ca. 10^{-5} M while the internal ATP concentration is ca. 10^{-3} M. An inhibitor of ATP production could reduce the internal ATP level by 90% without significantly affecting transport. (The inhibited level of ATP will still be 10 times K_m, allowing transport to operate at about 90% of its V_{max} [14].) The obvious conclusion (that ATP is not directly involved) would be incorrect. (ii) ATP is not directly involved in transport, but similar electron transport processes power both ATP synthesis and membrane transport. A proton-conducting inhibitor of electron transport at some specific concentration might well reduce the cellular ATP level and the activity of a permease by nearly the same amount. In spite of the positive correlation, the obvious conclusion (that ATP directly drives transport) would be incorrect.

Our results do not preclude other long-term

TABLE 2. *Effect of pyrithione on various transport systems*

Nutrient depletion condition	Transport system derepressed	Substrate tested (concn)	% Inhibition of transport: *P. notatum*	% Inhibition of transport: *P. chrysogenum*
−Sulfur	Sulfate	$^{35}SO_4^{2-}$ (10^{-5} M)	91	80
	Choline-*O*-sulfate	Choline-*O*-[^{35}S]sulfate (10^{-5} M)	77	
	Methionine	[^{3}H]methionine (10^{-4} M)	56	55
−Nitrogen	General amino acid	[^{14}C]phenylalanine (10^{-4} M)	70	75
		[^{14}C]valine (10^{-4} M)		61
		[^{3}H]methionine (10^{-4} M)	77	74
	Ammonium	[^{14}C]methylamine (2×10^{-4} M)	95	94
−Carbon	General amino acid	[^{14}C]phenylalanine (10^{-4} M)	56	48
		[^{14}C]valine (10^{-4} M)		42
	Glucose	[^{14}C]glucose (2.5×10^{-4} M)	52	59
−Phosphate	Phosphate	$^{32}P_i$ (2.5×10^{-4} M)		87

effects of pyrithione such as the reduction of disulfide bonds of intracellular proteins. Additionally, the suppression of α-keto acid excretion (Fig. 6) hints at a possible antitransaminase activity of pyrithione, thereby supporting the suggestion that pyrithione may act as a pyridoxal antimetabolite (5).

The relationship between the ability of pyrithione to inhibit membrane transport and the antidandruff activity of the compound is not clear. A discussion of the etiology of dandruff is beyond the scope of this article, but it seems reasonable that inhibition of the growth of scalp microflora might be involved in the efficacy of pyrithione. In this regard it is noteworthy that a number of antidandruff and antifungal topical agents are either weak, lipid-soluble acids (e.g., salicylic, propionic, benzoic, sorbic, and undecylenic acids) or compounds that could produce weak acids upon hydrolysis (e.g., tolnaftate) or reaction with skin protein thiols (e.g., sulfur, selenium sulfide).

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